

Bax Deficiency Partially Corrects Interleukin-7 Receptor α Deficiency

Annette R. Khaled,¹ Wen Qing Li,¹ Jiaqiang Huang,¹ Terry J. Fry,² Amr S. Khaled,³ Crystal L. Mackall,² Kathrin Muegge,⁴ Howard A. Young,⁵ and Scott K. Durum^{1,6}

¹Laboratory of Molecular Immunoregulation
Center for Cancer Research
National Cancer Institute – Frederick
Frederick, Maryland 21702

²Pediatric Oncology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

³Department of Pathology
University of Miami
Miami, Florida 33136

⁴Intramural Research Support Program
SAIC Frederick

⁵Laboratory of Experimental Immunology
Center for Cancer Research
National Cancer Institute – Frederick
Frederick, Maryland 21702

Summary

The requirement for cytokines in hematopoiesis is partly attributable to the protection of cells from apoptosis. Since IL-7 is required for normal T cell development, we evaluated the role of Bax *in vivo* by generating mice deficient in both Bax and the IL-7 receptor α chain (IL-7R). Starting at birth, we observed complete recovery of all stages of $\alpha\beta$ thymocyte development up to 4 weeks of age. However, by 12 weeks of age, thymic cellularity had reverted to that of mice deficient in IL-7R alone. The BH3 only proteins, Bad and Bim, were also part of the death pathway repressed by IL-7. Thus, in young mice, Bax emerges as an essential protein in the death pathway induced by IL-7 deficiency.

Introduction

A complex thymic microenvironment supports the growth, maturation, and selection of thymocytes. One essential component is interleukin-7 (IL-7) (Namen et al., 1988), a product of thymic stromal cells. Inactivating the gene for IL-7 (von-Freeden-Jeffrey et al., 1995) or either component of the IL-7 receptor, the α chain (IL-7R) (Peschon et al., 1994) or the γ_c chain (Cao et al., 1995), severely reduced thymic cellularity with an early block at the pro-T2 to pro-T3 stage (Hofmeister et al., 1999).

Several lines of evidence suggested that part of the effect of IL-7 was to promote survival of pro-T cells, and this “trophic” effect of IL-7 was originally attributed to inducing expression of the antiapoptotic protein Bcl-2 (Candeias et al., 1997a). Overexpression of Bcl-2 res-

cued T cell development in IL-7R^{-/-} mice (Maraskovsky et al., 1997; Akashi et al., 1997). However, forced expression of Bcl-2 did not rescue B lymphopoiesis (Kondo et al., 1997; Maraskovsky et al., 1998) or $\gamma\delta$ T cells (Nakajima and Leonard, 1999).

Bcl-2 is the founding member of a family of anti- and proapoptotic proteins whose interactions regulate cell death. The proapoptotic protein, Bax, originally discovered as a protein that coimmunoprecipitated with Bcl-2, is activated by various death stimuli and induces apoptosis (Oltvai et al., 1993). Bax-deficient mice proved viable and showed increased thymocytes (1.6-fold) and B cells (Knudson et al., 1995). Deletion of Bax prevented the apoptosis and thymic hypoplasia occurring in Bcl-2 deficient mice, suggesting that Bcl-2 functions to inhibit Bax in thymocytes (Knudson and Korsmeyer, 1997).

Neurons show some parallels with pro-T cells with regard to the role of Bax in trophic responses (Knudson et al., 1995). Primary neurons require the trophic factor NGF for survival in culture, and Bax^{-/-} neurons survive in the absence of NGF (Deckwerth et al., 1996). In TrkA^{-/-}/Bax^{-/-} or NGF^{-/-}/Bax^{-/-} mice, neuronal growth of proximal neurons was restored compared to TrkA^{-/-} or NGF^{-/-} mice, but production of neuronal processes remained impaired, with defects in sensory stimulation suggesting functions for NGF beyond inhibition of Bax-mediated death (Patel et al., 2000).

In the IL-7 response of pro-T cells, Bax was implicated as a death-promoting factor: Bax was detected under apoptotic conditions when the levels of Bcl-2 declined (Kim et al., 1998). Using an IL-7-dependent thymic cell line, we observed that IL-7 withdrawal induced a transient intracellular alkalization, mediating the mitochondrial translocation of Bax, and that antisense oligodeoxynucleotides to Bax protected cells from death (Khaled et al., 1999, 2001b).

To evaluate the role of Bax as a death mediator in pro-T cells deprived of an IL-7 signal, we generated mice with deficiencies in both the specific receptor for IL-7 (IL-7R) and Bax. Like in the NGF^{-/-}/Bax^{-/-} mice discussed above, Bax deficiency protects cells from death due to cytokine loss. The results show that Bax is a key mediator of the arrested thymocyte development in young mice deprived of a signal from the IL-7 receptor, yet reveal the presence of additional Bax-independent death factors in adult animals, suggesting that a complexity of apoptotic factors regulate death induced by loss of IL-7.

Results

Bax Deficiency Restores Thymocyte Development in IL-7R^{-/-} Mice

IL-7R^{-/-} mice show a striking loss of thymocytes attributed to the death of IL-7-dependent pro-T cells (Peschon et al., 1994). To determine if the apoptotic protein Bax mediated this cell death, we bred mice lacking expression of IL-7R and Bax. In Figure 1, anti-CD3 staining of mouse thymus sections from pairs of 8-week-

⁶ Correspondence: durums@mail.ncifcrf.gov

Anti-CD3 Staining

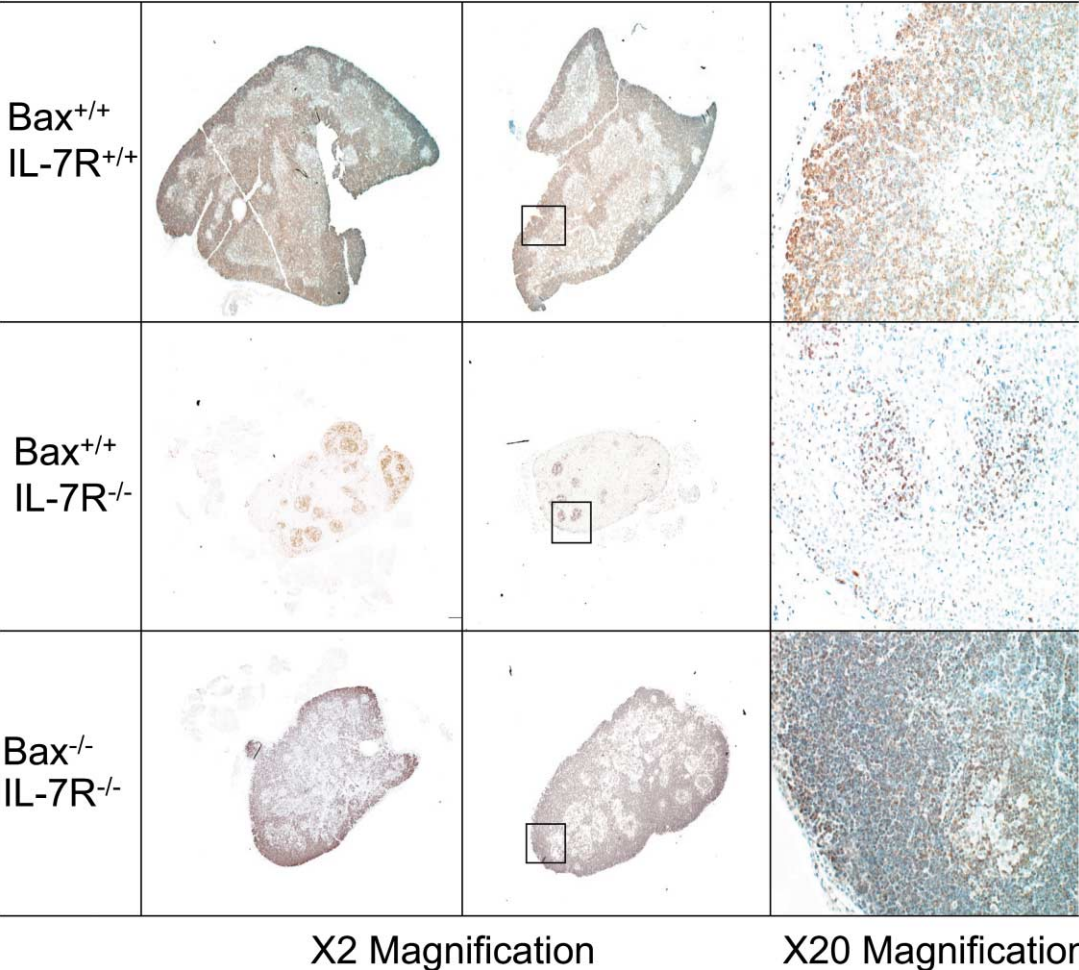


Figure 1. Bax Deficiency Restores T Cell Development in IL-7R-Deficient Mice
Mouse thymus sections from pairs of 8-week-old wt, IL-7R^{-/-}-deficient, and IL-7R^{-/-}/Bax^{-/-} mice were stained for anti-CD3 as described in Experimental Procedures. At X2 magnification, the recovery of normal thymic architecture is evident in the double-deficient mice (lower left panels) in comparison to the IL-7R^{-/-} mice (middle left panels). At X20 magnification, CD3⁺ T cells are seen in the cortex of wt (top right panel) and restored in the double-deficient mice (bottom right panel), but not in IL-7R^{-/-} mice (middle right panel).

old wt, IL-7R^{-/-}, and IL-7R^{-/-}/Bax^{-/-} mice is shown. Normal thymus from two wt mice exhibited extensive anti-CD3 staining for thymocytes in the outer cortex and less in the medulla. Thymus sections from Bax^{-/-} mice were similar in appearance to wt mice (data not shown). Thymuses from two IL-7R^{-/-} mice contained few thymocytes, lacked an organized cortex or medulla, and consisted predominantly of epithelial cells. “Leaky” development of thymocytes does occasionally occur in IL-7R^{-/-} mice; an example of a patch of CD3⁺ cells is enlarged in Figure 1. This leakiness was observed in two of seven IL-7R^{-/-} mice.

In contrast to the reduction in thymic cellularity seen in the IL-7R^{-/-} mice, IL-7R^{-/-}/Bax^{-/-} mice had significant thymocyte production. Displayed in Figure 1 are thymuses from two double-deficient mice, exhibiting abundant anti-CD3 staining in the cortex and medullary areas. Some differences from wt were apparent: thymuses

from the double-deficient mice were smaller and the medulla was divided into islets. In the absence of IL-7, this decrease in size of the thymus was not likely to be caused by thymic involution due to premature age. Older thymuses display volume increases in perivascular space, later replaced by adipose tissue, an architecture not apparent in the IL-7R^{-/-}/Bax^{-/-} mice. Higher power magnifications (Figure 1) reaffirmed that deletion of Bax restored significant development of CD3⁺ thymocytes in IL-7R^{-/-} mice.

We then examined the major thymocyte subsets in IL-7R^{-/-}/Bax^{-/-} mice. Thymocytes from wt mice (8 week old) consisted of double-positive (DP) CD4⁺CD8⁺ cells (88.4%) and single-positive (SP) CD4⁺ (32.6%) and CD8⁺ (16.7%) cells as shown in Figure 2A. In contrast, IL-7R^{-/-} mice (8 week old), with one or two normal Bax alleles, contained few thymocytes. Thymocytes from the IL-7R^{-/-}/Bax^{-/-} mice (8 week old) displayed a profile

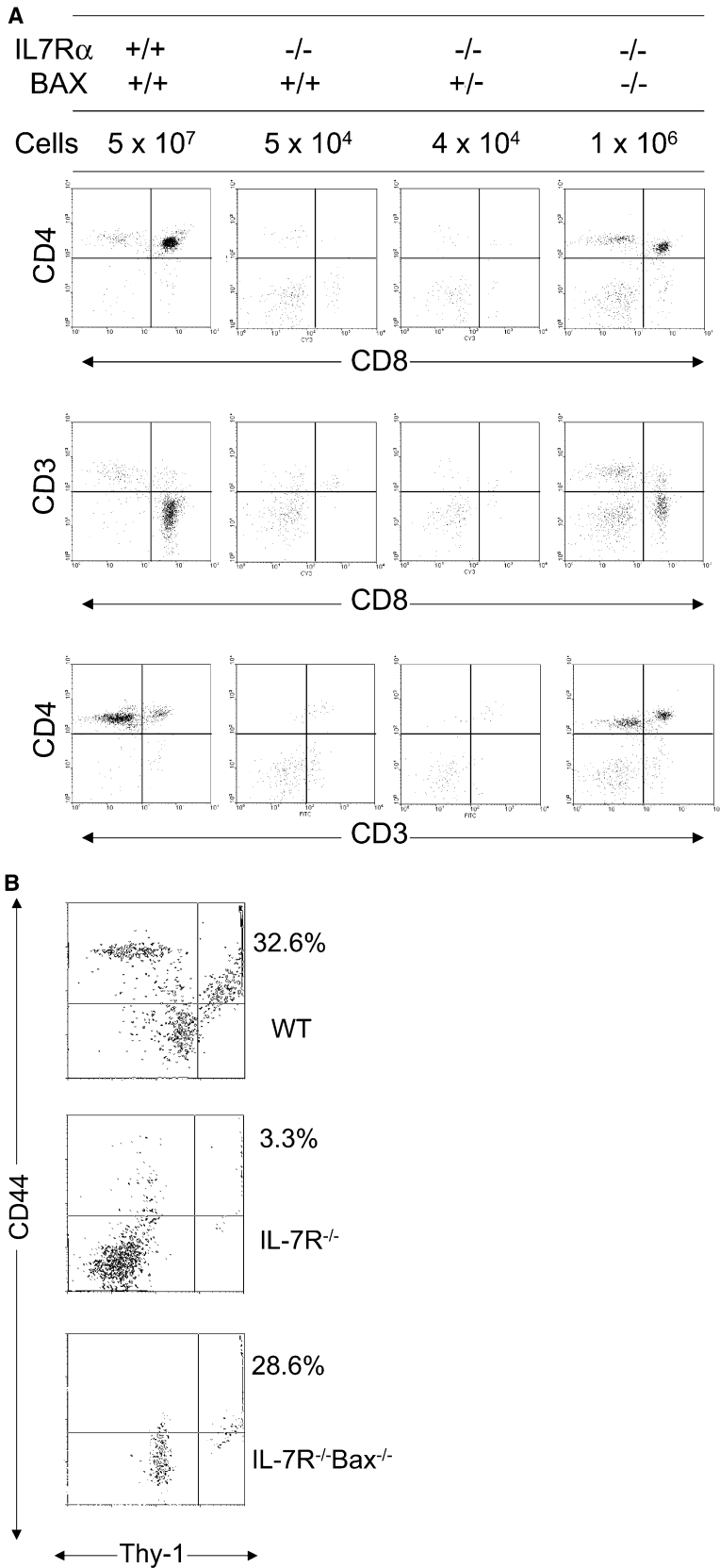


Figure 2. Bax Deficiency Restores Development of DP and SP T Cells, as Well as Thy-1 $^{+}$ CD44 $^{+}$ TN T Cells in an IL-7R $^{-/-}$ Thymus

(A) Thymocytes were isolated from 8-week-old wt, IL-7R $^{-/-}$, and IL-7R $^{-/-}$ /Bax $^{-/-}$ mice, stained with anti-CD4, anti-CD8, and anti-CD3, as described in Experimental Procedures, and analyzed by flow cytometry. Thymic cellularity for each genotype is shown at the top of each panel. The thymus from wt mice was primarily composed of CD4 $^{+}$ CD8 $^{+}$ DP cells and mature CD3 $^{+}$ CD4 $^{+}$ or CD8 $^{+}$ T cells. In the absence of IL-7R, very few cells stained positive for CD4 or CD8. However, in IL-7R $^{-/-}$ /Bax $^{-/-}$ mice, thymocytes recovered were CD4 $^{+}$ CD8 $^{+}$ as well as CD3 $^{+}$. Mice heterozygous for Bax did not show restored development in the absence of IL-7R. Shown is a representative result of four similar experiments.

(B) Thymocytes from 4-week-old wt, IL-7R $^{-/-}$, and IL-7R $^{-/-}$ /Bax $^{-/-}$ mice were stained and analyzed by flow cytometry as described in Experimental Procedures. Thymocytes that stained positive with anti-CD3-FITC, anti-CD4-FITC, and anti-CD8-FITC were gated out; displayed are TN pro-T cells (approximately 2%–3% of total thymocytes), distinguished by staining with anti-CD44-PE and Thy-1-CyChrome. Because of the abundance of Thy-1 on the cell surface, the intensity of the staining signal was very bright, forcing the Thy-1 $^{+}$ cells off the scale when analyzed. The proT1-2 population (Thy-1 $^{+}$ CD44 $^{+}$) represented 32.6% of the TN cells in wt mice, were reduced to 3.3% in IL-7R $^{-/-}$ mice, but were restored in the IL-7R $^{-/-}$ /Bax $^{-/-}$ mice to levels (28.6%) approaching that of wt. Shown is a representative experiment of two.

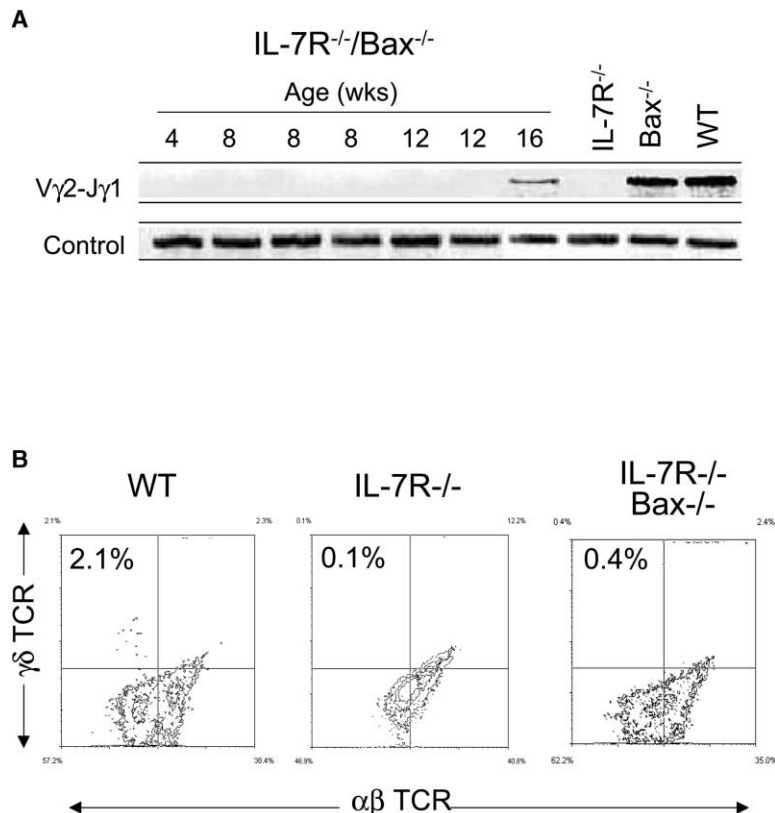


Figure 3. Bax Deficiency Does Not Restore Rearrangement of the TCR γ Locus or Development of Splenic $\gamma\delta$ T Cells

(A) Rearrangement of the TCR γ locus was evaluated by PCR with specific primers amplifying the region between V γ 2 and J γ 1, as described in Experimental Procedures. A PCR product is detected only when these two regions are joined. As control, a PCR product was amplified from the V γ region, which could be detected whether or not rearrangement occurred. No rearrangement of the TCR γ locus was observed in the absence of an IL-7 signal in splenocytes from Bax^{-/-} mice up to 12 weeks of age. A small amount of rearrangement was detected in splenocytes from Bax^{-/-}/IL-7R^{-/-} mice at 16 weeks of age, likely due to TCR γ rearrangement in surviving $\alpha\beta$ T cells.

(B) The generation of $\gamma\delta$ T cells was assessed by flow cytometry, staining with antibodies for TCR $\gamma\delta$ or, as control, TCR β , as described in Experimental Procedures. No $\gamma\delta$ T cells were detected in splenocytes from IL-7R^{-/-} mice, even in the absence of Bax. Shown is a representative experiment of three performed.

approaching that of wt, containing DP cells (50.3%) and SP CD4⁺ (40.9%) and CD8⁺ (28.6%) cells. High levels of CD3 are a marker of mature SP cells and more mature DP cells. As shown in Figure 2A, development of these more mature thymocyte subsets occurred in the double-deficient mice and was slightly increased compared to wt. The 2:1 ratio of SP CD4 to CD8 cells was also restored in the double-deficient mice shown. Evaluation of other IL-7R^{-/-}/Bax^{-/-} double-deficient mice, from 4–8 weeks old (data not shown), occasionally revealed an imbalance favoring CD4⁺ cells. This could occur because IL-7 promotes the generation of CD8 cells (Brugnera et al., 2000), probably through a Bax-independent mechanism. Therefore, in the absence of IL-7 signaling, Bax deficiency can restore normal thymic development of DP cells as well as mature SP thymocytes expressing high levels of CD3⁺.

To determine whether Bax deficiency restored pro-T cells in IL-7R^{-/-} mice, CD3⁺, CD4⁺, and CD8⁺ cells were gated out and the remaining triple-negative (TN) thymocytes analyzed for Thy-1⁺CD44⁺ cells, which include the pro-T1 and -T2 populations. Shown in Figure 2B is a representative experiment in which 32.6% of TN cells from a wt thymus were Thy-1⁺CD44⁺, while only 3.3% of TN cells from an IL-7R^{-/-} thymus were Thy-1⁺CD44⁺. Bax deficiency significantly restored the frequency of TN cells that were Thy-1⁺CD44⁺, with 28.6% of such cells recovered. This suggested that loss of Bax protected these early progenitor T cells from deprivation of IL-7 receptor signals, permitting them to develop into the later IL-7-independent thymocyte stages (DP and SP). It should be noted that a population of Thy-1⁺CD44⁺

cells were evident in the wt thymus, but not in the IL-7R^{-/-} thymus or IL-7R^{-/-}/Bax^{-/-} thymus. We believe these to be nonlymphoid cells, likely thymic macrophages derived from early lymphocyte progenitors—a process that requires IL-7 (Lee et al., 2001).

Bax Deletion Does Not Restore Normal Rearrangement of the TCR γ Locus, $\gamma\delta$ T Cell Development, or B Lymphopoiesis in IL-7R α ^{-/-} Mice

IL-7 deficiency interrupts $\gamma\delta$ T cell development, in part by blocking rearrangement of the TCR γ locus (Maki et al., 1996; Candeias et al., 1997a, 1997b). Various lines of evidence suggest that the effect of IL-7 on this gene rearrangement process is through opening the chromatin in the region of the TCR γ locus (Candeias et al., 1997b; Durum et al., 1998), rather than protection from cell death. For example, enforced expression of the anti-apoptotic protein, Bcl-2, in mice deficient for IL-7 signaling did not restore $\gamma\delta$ T cell development (Nakajima and Leonard, 1999). We evaluated IL-7R^{-/-}/Bax^{-/-} mice using specific primers to detect TCR γ locus rearrangement by PCR methods previously described (Durum et al., 1998). Shown in Figure 3A is the result of one such experiment detecting TCR γ locus rearrangement in splenic cells from wt mice but not IL-7R^{-/-} mice. As shown, TCR γ locus rearrangement was not restored in spleen cells from the IL-7R^{-/-}/Bax^{-/-} mice, although a low level of rearrangement could be detected in mice after 16 weeks of age (Figure 3A). This result is consistent with the other cited evidence that the effect of IL-7 on rearrangement of the TCR γ locus is not based on protection from apoptosis. Approximately

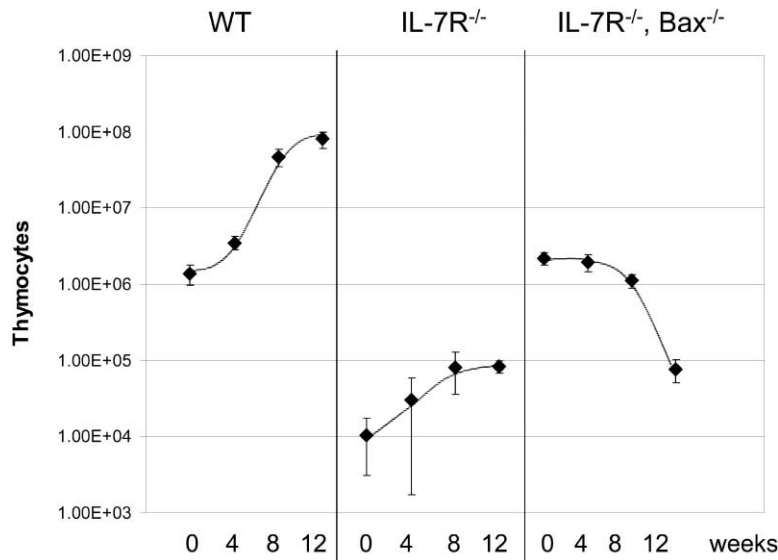


Figure 4. Bax Deletion Rescues Thymic Development in IL-7R^{-/-} Mice Early in Life, Then Declines with Age

Thymic cellularity in wt, IL-7R^{-/-}, and IL-7R^{-/-}/Bax^{-/-} mice was determined microscopically at birth or various ages thereafter. At birth, wt and IL-7R^{-/-}/Bax^{-/-} mice had equivalent numbers of thymocytes (between $1-2 \times 10^6$ cells/thymus), while the IL-7R^{-/-} mice had fewer than 10^4 cells/thymus. However, with increasing age the IL-7R^{-/-}/Bax^{-/-} thymic cellularity declined to that of IL-7R^{-/-} mice. Each age group represents two to four animals evaluated.

half of $\alpha\beta$ T cells have a rearranged TCR γ locus; thus in normal thymocytes, most of the signals for rearrangement of this locus come from the more plentiful $\alpha\beta$ cells, rather than the infrequent $\gamma\delta$ cells. We presume therefore that the low level of rearrangement detected in older double-deficient mice represents $\alpha\beta$ T cells that survive in the Bax-deficient mice. In support of this conclusion, no $\gamma\delta$ T cells were detected in the thymus (data not shown) or spleen from double-deficient mice even after 12 weeks of age (Figure 3B); earlier ages were also examined and showed no $\gamma\delta$ T cell development (not shown). These results do not rule out the (quite plausible) possibility that IL-7 could promote $\gamma\delta$ T cells in two ways, first through rearrangement of the TCR γ locus and second through an antiapoptotic mechanism.

We also examined the effects on peripheral lymphocytes in IL-7R^{-/-}/Bax^{-/-} double-deficient mice. We previously observed that peripheral lymphocytes accumulate in IL-7R^{-/-} mice, and here we note that Bax deletion only modestly augmented this accumulation. At 4 weeks of age, the splenic cellularity in the IL-7R^{-/-}/Bax^{-/-} double-deficient mouse was a log-fold higher than in the IL-7R^{-/-} mice (6×10^6 splenocytes compared to 1.6×10^5 splenocytes), but much lower than wt (1.5×10^7). However, at 8 weeks, IL-7R^{-/-} mice averaged 3×10^6 splenocytes and IL-7R^{-/-}/Bax^{-/-} double-deficient mice averaged 5.1×10^6 splenocytes while wt mice averaged 2.8×10^7 splenocytes. Thus, from 4–8 weeks, only slight increases in total splenic cellularity were observed in the double-deficient mice over that of IL-7R^{-/-} mice. The percentages of splenic T or B lymphocytes were not increased in double-deficient mice (data not shown), indicating that the IL-7 requirement for homeostasis of peripheral T lymphocytes and B lymphopoiesis is not based solely on repression of Bax.

Bax Deletion Restores Thymic Content in IL-7R^{-/-} Mice Early in Life, Then Declines with Age

Bax deficiency restored significant thymic development in IL-7R^{-/-} mice. To determine the extent of this restoration, thymic number was determined in mice from birth through 12 weeks and results shown in Figure 4. At birth,

wt thymus contained an average of 1.2×10^6 cells, whereas IL-7R^{-/-} thymus contained less than 10^4 cells. IL-7R^{-/-}/Bax^{-/-} thymus contained an average of 2×10^6 cells, slightly more than wt. Bax deficiency substantially compensated for IL-7 deficiency up through 4 weeks of age. At this time, wt mice averaged 3.5×10^6 cells/thymus, IL-7R^{-/-} mice averaged 3.0×10^4 cells/thymus, and the IL-7R^{-/-}/Bax^{-/-} averaged 2.1×10^6 cells/thymus. However, by 8 weeks, when thymic expansion is measurable, the wt thymus increased to 4.2×10^7 cells/thymus, but not the IL-7R^{-/-}/Bax^{-/-} thymus, which averaged only 1.0×10^6 cells/thymus, only a log-fold greater than the IL-7R^{-/-}. By 12 weeks, the double-deficient thymus contained no more thymocytes than IL-7R^{-/-}. Therefore, Bax deficiency protected thymocytes from IL-7R deficiency until about 4 weeks of life, after which additional Bax-independent requirements for IL-7 dominated.

Fetal Liver Cells or Adult Bone Marrow Cells from Bax-Deficient Mice Fail to Reconstitute an IL-7-Deficient Thymus but Do Reconstitute Fetal Thymuses

To determine if the age-dependent protection afforded by Bax deficiency was an intrinsic characteristic of fetal cells, we compared the capacity of fetal liver (FL) cells and adult bone marrow (BM) cells from a Bax^{-/-} mouse to reconstitute an IL-7-deficient thymus. If cells of fetal origin only expressed the death protein, Bax, while adult cells expressed multiple apoptosis inducers (for example the death protein, Bak), then Bax^{-/-} FL cells but not adult Bax^{-/-} BM cells could reconstitute an adult thymus in the absence of IL-7. Summarized in Table 1A are the results of several reconstitution experiments, using as recipients Rag^{-/-} mice (positive controls) and Rag^{-/-}/IL-7^{-/-} mice. Adult BM cells from either Bax^{-/-} mice or Bax^{+/+} mice did not reconstitute the IL-7-deficient recipients. Interestingly, although BM cells from Bax^{+/+} mice did reconstitute the Rag-deficient thymus, Rag^{-/-} recipients receiving BM cells from Bax^{-/-} mice died after 7 days, perhaps from graft versus host disease.

Table 1. Bax Deficiency Rescues Development of IL-7R^{-/-} Thymocytes in Fetal but Not Adult Thymus(A) Bax-Deficient Fetal Liver (FL) Cells or Adult Bone Marrow (BM) Cells Fail to Reconstitute an Adult Rag^{-/-}/IL-7^{-/-} Thymus after 4 Weeks

Donor	Recipients ^a	Total Thymocytes
Bax ^{-/-} BM	Rag ^{-/-} /IL-7 ^{-/-}	1.5 × 10 ⁵
	Rag ^{-/-} /IL-7 ^{-/-}	1.0 × 10 ⁴
Bax ^{+/+} BM	Rag ^{-/-} /IL-7 ^{-/-}	1.0 × 10 ⁴
	Rag ^{-/-} /IL-7 ^{-/-}	1.0 × 10 ⁴
Bax ^{-/-} BM	Rag ^{-/-}	(Died)
	Rag ^{-/-}	(Died)
Bax ^{+/+} BM	Rag ^{-/-}	1.12 × 10 ⁷
	Rag ^{-/-}	1.03 × 10 ⁷
Bax ^{-/-} FL	Rag ^{-/-} /IL-7 ^{-/-}	1.0 × 10 ⁵
	Rag ^{-/-} /IL-7 ^{-/-}	1.5 × 10 ⁵
Bax ^{+/+} FL	Rag ^{-/-} /IL-7 ^{-/-}	1.0 × 10 ⁵
	Rag ^{-/-} /IL-7 ^{-/-}	2.0 × 10 ⁵
Bax ^{-/-} FL	Rag ^{-/-}	5.60 × 10 ⁷
	Rag ^{-/-}	3.96 × 10 ⁷
Bax ^{+/+} FL	Rag ^{-/-}	3.80 × 10 ⁷
	Rag ^{-/-}	3.69 × 10 ⁷

(B) Bax^{-/-}/IL-7R^{-/-} Adult Bone Marrow (BM) Cells Can Reconstitute a Fetal Thymic Organ

Donor	Recipient	Total Thymocytes/Lobe ^b
None	Unirradiated fetal thymus	8.2 × 10 ⁴
None	Irradiated fetal thymus	1.2 × 10 ⁴
Bax ^{+/+} /IL-7R ^{+/+}	Irradiated fetal thymus	7.1 × 10 ⁴
Bax ^{-/-} /IL-7R ^{+/+}	Irradiated fetal thymus	4.9 × 10 ⁴
Bax ^{+/+} /IL-7R ^{-/-}	Irradiated fetal thymus	1.3 × 10 ⁴
Bax ^{-/-} /IL-7R ^{-/-}	Irradiated fetal thymus	4.9 × 10 ⁴

^a Two individual recipients are shown per each donor.^b Five to eight fetal thymic lobes are shown per each donor.

Next, donor FL cells were injected into IL-7-deficient hosts. Neither Bax^{-/-} nor Bax^{+/+} FL cells were able to reconstitute the recipient mice (Rag^{-/-}/IL-7^{-/-}) in the absence of IL-7 but did reconstitute in the presence of IL-7 (Rag^{-/-}). Bax deficiency, therefore, did not permit FL cells to undergo thymic differentiation in an adult mouse in the absence of IL-7; hence, there is no intrinsic difference in fetal versus adult thymocytes. Fetal liver cells are armed with the same death inducers as adult bone marrow cells. Instead, the difference appeared to lie between the adult versus fetal environments.

To directly test whether the fetal thymic environment was amenable to reconstitution by Bax-deficient BM cells in the absence of IL-7, irradiated fetal thymuses were repopulated in organ culture with BM cells from either an adult (12 weeks old) wt mouse, a Bax^{-/-} mouse, an IL-7R^{-/-} mouse, or a Bax^{-/-}/IL-7R^{-/-} mouse. In contrast to the results observed with adult thymuses, Bax deficiency was able to restore reconstitution of irradiated fetal thymuses in the absence of IL-7 signaling, with almost a 4-fold enhancement of thymic expansion (Table 1B). Therefore, we propose that in the fetal environment loss of IL-7 triggers a death pathway requiring Bax. In the adult thymus, extrinsic stresses (for example, steroids or death ligands) are present that induce death pathways independent of Bax, but inhibited by IL-7.

Bcl-2 Transgene Expression or Bax Deficiency Protects Adult Pro-T Cells from IL-7 Withdrawal-Induced Death

Having shown that, in young mice, Bax deletion restored thymocyte survival in the absence of IL-7, we examined,

in greater detail, the role of Bax, and its inhibitor, Bcl-2, in death induced by IL-7 withdrawal in adult cells. TN thymocytes from adult Bax^{-/-} mice and wt controls were cultured in vitro for 3 days with or without IL-7. Bax deletion protected adult TN thymocytes from IL-7 deprivation as shown in Figure 5A, with greater than 30% increased viability apparent in the absence of IL-7 after 48 and 72 hr in culture. Bax deletion also protected TN cells from IL-7-independent death in vitro. Hence, Bax deficiency has the potential to protect adult pro-T cells from death mediated by IL-7 withdrawal, further confirming that Bax is an essential death effector triggered in the absence of IL-7.

Transgenic expression of the antiapoptotic protein, Bcl-2, has also been shown to restore thymocyte development in IL-7R^{-/-} mice. To confirm this, we isolated TN cells from adult Bcl-2 tg mouse and observed prolonged survival of pro-T cells in the absence IL-7 in vitro (Figure 5B). Note that Bcl-2 overexpression during IL-7 withdrawal was more protective than deficiency in Bax. Others have shown that transgenic expression of Bcl-2 in lymphocytes in IL-7R^{-/-} mice was protective up to 16 weeks (E. Maraskovsky and A. Strasser, personal communication), while Bax deficiency protected only up to 8 weeks. This suggests that the antiapoptotic activity of Bcl-2, during IL-7 withdrawal, extends to other apoptotic inducers, in addition to Bax, present in the adult animal.

Bax-Independent Death Pathways: PI3-Kinase and Bad, Bim, and Bak

To begin the identification of novel death mediators induced in adult thymocytes, we evaluated PI-3 kinase,

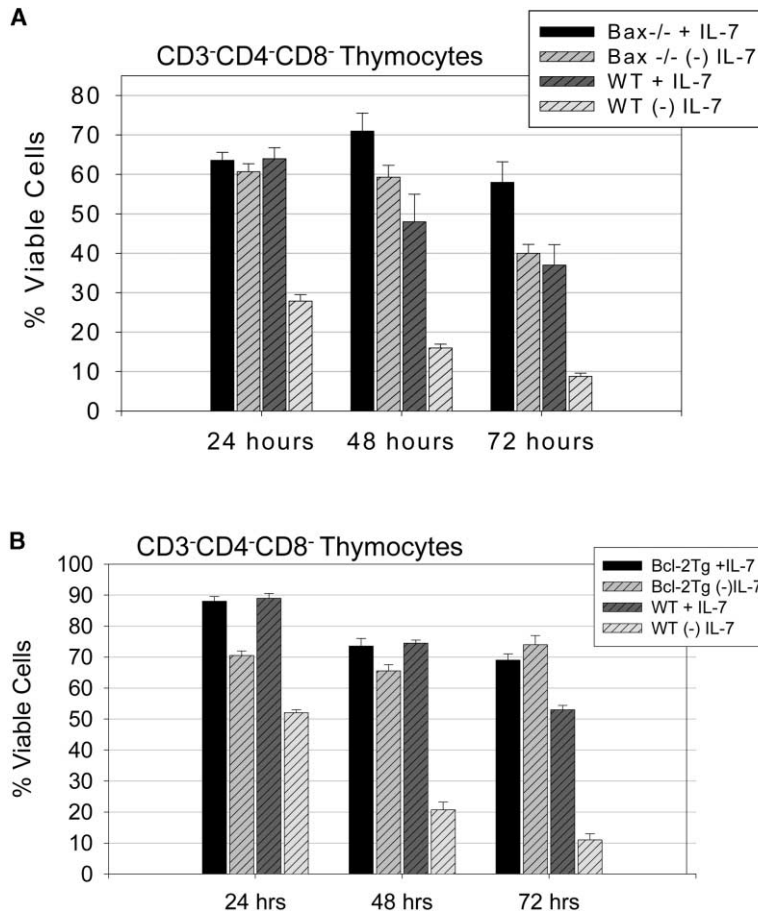


Figure 5. Bax Deficiency or Forced Expression of Bcl-2 Protects Pro-T Cells from IL-7 Withdrawal

TN thymocytes from wt, Bax^{-/-}, or Bcl-2tg mice were isolated by negative selection with magnetic beads as described in Experimental Procedures. TN cells were placed in cell culture with or without IL-7 for 3 days, and cell number and viability determined microscopically by trypan blue exclusion.

(A) Total thymic pro-T cells from wt or Bax^{-/-} mice were cultured for 3 days with or without IL-7. Bax deficiency protected pro-T cells from IL-7 withdrawal-induced death as well as from spontaneous cell death.

(B) Pro-T cells from either wt or a Bcl-2 transgenic mouse were placed in cell culture with or without IL-7 for 3 days. Pro-T cells expressing the Bcl-2 transgene were protected from cell death due to IL-7 withdrawal through the 3 days evaluated in the assay. Shown are representative experiments of three similarly performed.

an essential component in the regulation of apoptotic proteins like Bad (del-Peso et al., 1997) and Bim (Dijkers et al., 2000). Data shown in Figure 6A are representative of several experiments demonstrating the effect of LY294002, a PI3-kinase inhibitor, on TN thymocytes from wt or Bax^{-/-} mice. Treatment with LY294002 preferentially killed pro-T2 (CD44⁺CD25⁺) and pro-T3 (CD44⁺CD25⁺) cells independent of Bax. The surviving CD44⁺CD25⁻ TN cells could be nonlymphoid cells.

Thymocytes from a Rag-1^{-/-} or SCID mouse are primarily pro-T2 and T3 cells (CD25⁺) since VDJ recombination and further T cell development is arrested. These immature thymocytes were cultured with or without IL-7 for 6 hr, and high expression of the proapoptotic proteins Bad and Bim was detected (Figure 6B). In particular, we observed that IL-7 promoted Bad phosphorylation in both pro-T cells (Figure 6B) and in an IL-7-dependent thymic cell line (D1) (Figure 6C), whereas loss of IL-7 caused the dephosphorylation (and likely) activation of Bad. Shown as a control, IL-3 induced Bad phosphorylation in an IL-3-dependent pro-B cell line, FL5.12A (Figure 6C). There are also additional IL-7-independent effects (for example, cell culture stress) that caused Bad dephosphorylation—most noticeable at the later time points (Figure 6B). Taken together, these results suggest that the PI3K-AKT-Bad pathway could be a death mechanism, possibly upstream of Bax (discussed below), or could inactivate Bcl-2, contributing to the death of adult pro-T cells following IL-7 withdrawal.

We also measured the levels of the three isoforms of the apoptotic protein, Bim, in pro-T cells and the D1 thymic cell line. Induction of Bim synthesis occurs in the absence of PI3 kinase-AKT signaling through the forkhead transcription factor, FKHR-L1 (Dijkers et al., 2002). This was evident in an IL-3-dependent pro-B cell line, FL5.12A, tested with or without IL-3 for 8 hr, in which Bim induction occurred during the loss of IL-3 (Figure 6C). However, the levels of Bim protein did not change in pro-T cells, with or without IL-7 (Figure 6B), nor in the thymic cell line, D1 (Figure 6C). Hence, IL-7 (unlike IL-3) does not appear to regulate the synthesis of Bim, and this apoptotic protein is always present in pro-T cells independent of IL-7.

The BH3 proteins Bad and Bim are capable of initiating apoptosis, and, in many cells, this requires Bax or Bak (Zong et al., 2001). Therefore, in the absence of Bax, IL-7 withdrawal-induced death in adult thymocytes could be due to Bak, another apoptotic protein similar to Bax. Although we detected *bak* mRNA in pro-T cells using an RNase protection assay (RPA) (data not shown), we could not detect Bak protein. Shown in Figure 6D is an overexposed blot demonstrating that Bak could be readily detected in HL-60 cells or mouse splenocytes but not in pro-T cells cultured with or without IL-7 for 6 hr. In contrast, Bax protein was more than abundant in pro-T cells (Figure 6D). Measurement of *bak* and *bax* mRNA levels by RPA, which showed both transcripts made constitutively, revealed no increases due to IL-7

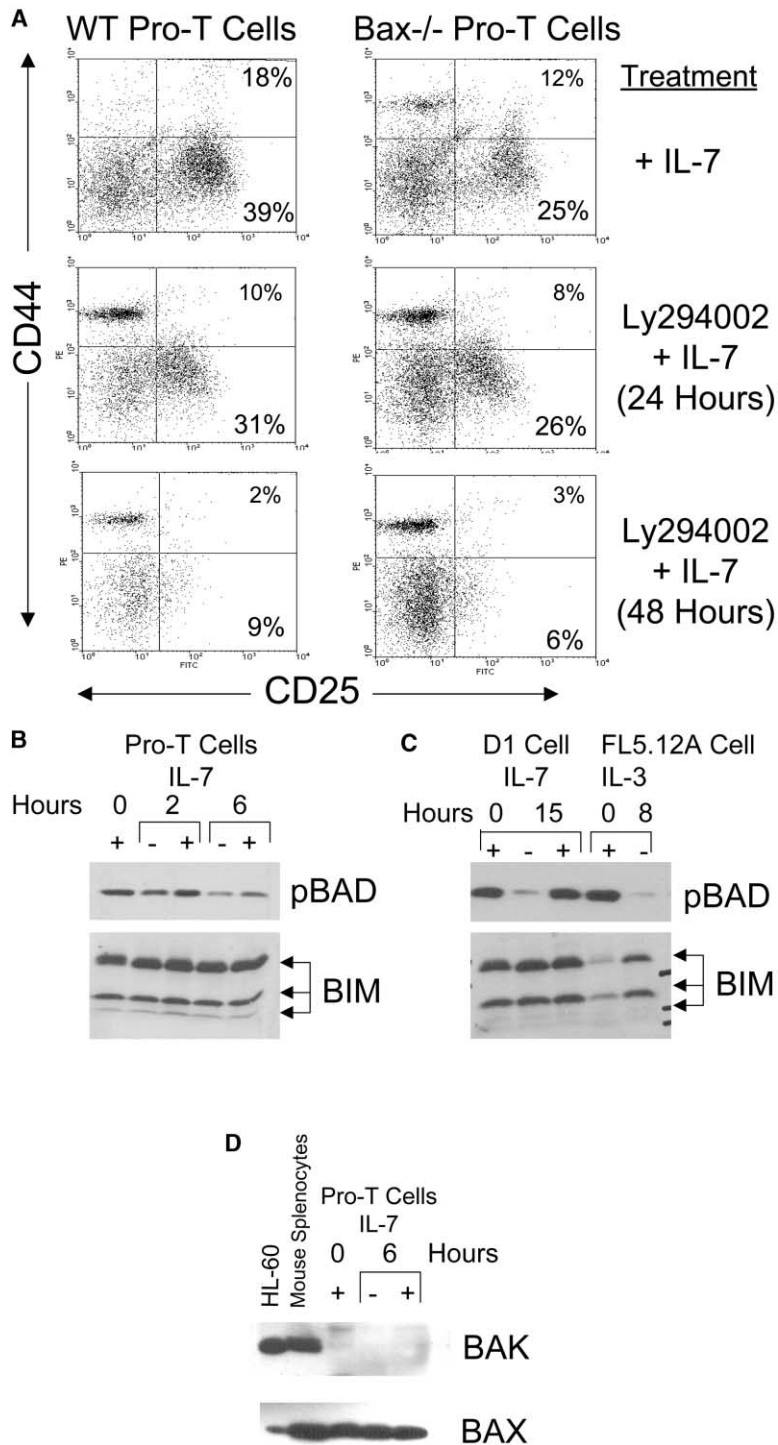


Figure 6. PI3-Kinase Regulates Bad and Bim, which are Implicated Upstream of Bax and Bak in Death Induced by Cytokine Deprivation

(A) PI3-kinase inhibitor kills CD25⁺ pro-T cells independent of Bax. TN thymocytes from either wt or Bax^{-/-} mice were isolated by negative selection with magnetic beads as described in Experimental Procedures. Cells were placed in cell culture with IL-7 and with or without 20 μ M LY294002, a PI3-Kinase inhibitor, for 2 days. Early pro-T cell subsets were identified by staining with anti-CD25-FITC and anti-CD44-PE through flow cytometry as described in Experimental Procedures. By 48 hr, the treatment with LY294002 killed most of the pro-T2 and -T3 (CD25⁺) cells from either the wt or Bax^{-/-} mice. Hence, functional PI3-kinase signaling is part of the survival mechanism for pro-T2 and -T3 cells.

(B–D) Thymocytes were isolated from SCID mice (shown) and repeated with Rag-deficient mice (not shown), since these would be pro-T2-T3 cells and dependent on IL-7 for survival. Thymocytes were placed in cell culture for 6 hr with or without IL-7 and a time 0 hr control included. Whole-cell lysates were made after incubation was completed and samples assessed for the protein levels of phosphorylated Bad and the three isoforms of Bim (B) and Bax and Bak (D). In addition, two cytokine-dependent cell lines were also assayed for phosphorylated Bad and Bim—an IL-7-dependent thymic cell line (D1) and an IL-3 dependent pro-B cell line (FL5.12A) (C). IL-7 regulates the levels of Bad phosphorylation in pro-T cells and the thymic cell line, D1, as does IL-3 in pro-B cells, but IL-7 does not induce the synthesis of Bim_{EL}, Bim_{IL}, or Bim_S (indicated by arrows) during withdrawal (B and C), while IL-3 does (C). No detectable levels of Bak protein in pro-T cells were observed in the overexposed blot shown (D) although measurable levels of Bak were detected in the control samples from HL60 and mouse splenocytes. In contrast, high levels of Bax protein were found in all lysates (D). A representative experiment is shown of two performed.

withdrawal in either pro-T cells or the IL-7-dependent D1 thymocyte line (data not shown). Because pro-T cells transcribe *bak*, it remains possible that a small amount of Bak protein is present in mitochondria, but below the level of detection in whole-cell lysates shown in Figure 6D, and that Bak contributes to cell death. Nonetheless, we find that Bax is highly expressed in pro-T cells, along with Bim and Bad, the latter of which is regulated through phosphorylation by IL-7, and that these apo-

ptotic factors could all contribute to cell death in the absence of IL-7.

Discussion

Defects in the signaling pathway of the IL-7 receptor are the major cause of severe combined immunodeficiency in man, and these effects are recapitulated in knockout mice (von-Frieden-Jeffrey et al., 1995;

Peschon et al., 1994). Part of the requirement for IL-7 is in promoting survival of pro-T cells. In the current study, we deleted the apoptotic protein, Bax, and restored thymocyte development in IL-7R^{-/-} mice. The IL-7R^{-/-}/Bax^{-/-} mice showed restoration of thymic cortex and medulla, containing DP and mature SP T cells. These effects were most apparent early in life; then, by 12 weeks of age, the double-deficient thymus reverted to the depleted state of the IL-7R^{-/-} thymus. Bax deletion restored significant development of T cells in the absence of IL-7, but we also show that a number of IL-7 functions are independent of repressing Bax. These IL-7 functions included protecting thymic development in older mice, peripheral expansion of T and B cells, inducing rearrangement of the TCR γ locus and generating $\gamma\delta$ T cells.

Previous studies using different knockout and transgenic mouse strains are consistent with our findings. IL-7 was shown to induce synthesis of Bcl-2 in pro-T cells (Kim et al., 1998), IL-7R^{-/-} defects were partly corrected with a Bcl-2 transgene (Kondo et al., 1997; Maraskovsky et al., 1997), and IL-7R^{-/-} defects partly resemble Bcl-2^{-/-} defects (Veis et al., 1993). While those studies suggested that an important response to IL-7 could be the synthesis of Bcl-2, they raised the following question: Why do these thymocytes need Bcl-2? As shown herein, protection from Bax is part of the answer.

The IL-7 receptor signaling complex recruits the Janus kinase, Jak3 (as do a number of cytokine receptors), and recently, a mouse was generated that lacked Bax and Jak3 (Wen et al., 2001). The reduced numbers of T cells in Jak3^{-/-} mice were corrected in Jak3^{-/-}/Bax^{-/-} mice, evaluated between 4–6 weeks of age, but peripheral T cell homeostasis was not normal. Retroviral introduction of Bcl-2 into Jak3^{-/-} bone marrow cells did restore T cell numbers (Wen et al., 2001). From their results, the authors concluded that Jak3-mediated regulation of Bax and Bcl-2 promotes survival during T cell development. However, since these double-deficient mice were only evaluated at an early age, it is unclear whether the protection afforded by Bax deficiency was long lasting. In our work, evaluating the loss of IL-7 signaling through disruption of the IL-7 receptor per se, we observed that Bax deletion protected thymopoiesis from birth through 4–8 weeks, but not thereafter (Figures 1–4). Because a Bcl-2tg appears to have a broader effect than Bax deletion (Figures 5A and 5B) with protection from IL-7 deprivation lasting at least up to 16 weeks in mice (Maraskovsky et al., 1997), it suggests that additional apoptotic Bcl-2 binding proteins are activated in adult thymocytes.

There are a number of pro-death members of the Bcl-2 family. Among these, Bak is most closely related to Bax, having a transmembrane domain. During drug-induced apoptosis, Bak is activated by exposing its amino terminus and binding Bcl-X_L (Griffiths et al., 2001). Mice deficient for Bak were developmentally normal (Lindsten et al., 2000); however, Bax^{-/-}/Bak^{-/-} mice had many developmental defects, with fewer than 10% of these mice surviving into adulthood (Lindsten et al., 2000). Lymphocytes from Bax^{-/-}/Bak^{-/-} mice were resistant to multiple apoptotic stimuli (Wei et al., 2001). Therefore, Bak would be a likely candidate for a death protein that kills independently of Bax in lymphocyte progenitors

deprived of IL-7. However, Bak not detectable in protein lysates from adult pro-T cells (Figure 6D) with no increases in Bak transcription detected during IL-7 withdrawal (data not shown). Its remains to be determined if pro-T cells express small but toxic amounts of Bak that can cause cell death in the absence of IL-7 or whether another death protein induces apoptosis in lieu of Bax or Bak.

In addition to Bax and Bak, other proapoptotic Bcl-2 family members include the multidomain protein, Bok, which is expressed in reproductive organs (Hsu et al., 1997) and not a candidate for lymphocyte apoptosis, and the “BH3” only proteins, Bid, Bad, and Bim. Bid-deficient mice were resistant to Fas-induced apoptosis, but had normal cell death when induced by other stimuli (Yin et al., 1999). Furthermore, the death of pro-T cells through IL-7 withdrawal is independent of Fas (Kim et al., 1998); therefore, Bid is an unlikely death inducer for pro-T cells.

In addition to repressing Bax, an additional IL-7 survival pathway could be through PI3-kinase, which regulates the death proteins, Bad and Bim. Targeted deletion of the catalytic subunit of PI3-kinase reduced thymic cellularity (Sasaki et al., 2000). The pro-life signal transduced through PI3-kinase leads to activation of AKT. AKT, in turn, phosphorylates the pro-death protein, Bad (del-Peso et al., 1997), which recruits 14-3-3 proteins, sequestering and inactivating Bad (Datta et al., 2000). Inhibition of PI3-kinase killed CD25⁺ T cells (Figure 6A). We show that these cells express high levels of Bad, that IL-7 promotes Bad phosphorylation, and that, following IL-7 withdrawal, Bad is dephosphorylated (Figures 6B and 6C) and targets mitochondria (W.Q.L., A.R.K., and S.K.D., unpublished data). IL-7 can activate PI3-kinase in lymphocytes (Venkitaraman and Cowling, 1994). A Bad transgene also perturbed T cell development (Mok et al., 1999), although a Bad-deficient mouse had no distinguishing phenotype (Downward, 1999).

AKT also inactivates a forkhead transcription factor (FKHRL1), through phosphorylation and retention in the cytosol via 14-3-3 proteins (Brunet et al., 1999). Upon loss of a survival signal, FKHRL1 is dephosphorylated and translocates to the nucleus, inducing the death protein, Bim (Dijkers et al., 2000). However, we found no changes in Bim protein levels with or without IL-7, suggesting that, unlike the induction of Bim shown to occur in the absence of IL-3 signaling (Figure 6C), IL-7 does not regulate Bim synthesis either in pro-T cells or an IL-7-dependent thymic cell line (Figures 6B and 6C). However, Bim could be a candidate for a death inducer in adult lymphocytes based on its role in killing mature peripheral T cells following withdrawal of cytokines: in CD8⁺ T cells, deletion of Bim protected about half of the cells from death following withdrawal of IL-7 in vitro (Bouillet et al., 2002).

A consensus model could be explained as follows. IL-7 stimulation: (1) induces synthesis of Bcl-2 and (2) represses Bax activation and promotes Bad phosphorylation and cytosolic sequestration. Removal of IL-7: (1) terminates synthesis of Bcl-2 (and perhaps other survival proteins), (2) inhibits the remaining Bcl-2 through Bad and Bim binding, rendering mitochondria vulnerable, and (3) activates Bax, which translocates to mitochondria and initiates the apoptotic cascade.

There is evidence for IL-7 effects in addition to promoting survival. Like the NGF effects on neurons, IL-7 appears to have functions beyond inducing Bcl-2 and repressing Bax. For example, IL-7 induces replication of cell lines. Metabolic dysfunction also results from IL-7 withdrawal. Recent work has shown that growth factors mediate survival through regulation of glucose uptake and metabolism (Vander et al., 2001). We have shown changes in cytosolic pH and mitochondrial ADP import accompany loss of cytokine signaling (Khaled et al., 2001a, 2001b). Could such aberrant metabolic functions contribute to the failure of Bax deletion to restore thymopoiesis in older mice? Perhaps, but it seems unlikely—glucose metabolism and ATP synthesis should be required in early thymopoiesis as well as in adult, and expression of a Bcl-2tg was protective up to 4 months in IL-7-deficient mice, well beyond the effects elicited by Bax deficiency.

Are lymphoid progenitors intrinsically different if derived from fetal liver compared to adult bone marrow? This was demonstrated in the requirement for Bcl-2 (Matsuzaki et al., 1997). Stem cells from Bcl-2^{-/-} fetal livers reconstituted thymopoiesis in irradiated recipients, whereas Bcl-2^{-/-} stem cells from adult bone marrow did not. However, in our study of IL-7 deficiency, we could not detect an intrinsic difference between fetal and adult stem cells, since neither Bax^{-/-} fetal liver cells nor adult bone marrow reconstituted the adult thymus in the IL-7^{-/-} recipient (Table 1A). Hence, both fetal and adult lymphoid stem cells are “armed” with the same death effectors.

We favor the hypothesis that the failure of Bax deletion to restore thymic development in older mice is due to extrinsic “stress” factors active in the adult but not in the young. This is supported by our experiments showing that, in the absence of IL-7, Bax^{-/-} BM cells could reconstitute a fetal thymus but not an adult thymus (Tables 1A and 1B). The engagement of death receptors, in particular CD95 or Fas, is essential in the elimination of activated T cells (Krammer, 2000) but does not appear to participate in death by IL-7 withdrawal (Kim, 1998). Another possibility is that glucocorticoids (GCs) in the adult environment trigger lymphocyte death, a process inhibited by Bcl-2. Various lines of support for this idea exist. For example, levels of GC are low during gestation (Vacchio and Ashwell, 2000) and increase rapidly after birth. GC-induced apoptosis is inhibited by Bcl-2 and is mediated through mitochondrial damage (Vacchio and Ashwell, 2000). Lastly, other signal transduction pathways, for example STAT5, can interrupt GC signaling (Biola et al., 2001).

Signals mediated through engagement of the IL-7 signaling complex promote the survival of T cell progenitors. In addition to the antiapoptotic protein, Bcl-2, we have shown that the proapoptotic protein Bax is integral to the cell death process suppressed by IL-7. By eliminating Bax, we demonstrated the involvement of other apoptotic pathways, countered by Bcl-2, highlighting Bad, normally regulated by the PI3-kinase-AKT pathway, and Bim. However, Bad and Bim are not thought to induce cell death without Bax or Bak (Zong et al., 2001). Hence, in the absence of Bax, Bak appears to be the most likely killer of adult thymocytes; however, Bak protein was undetectable in adult pro-T cells (Figure

6D), suggesting that other apoptotic proteins could also participate. Major remaining questions concern the IL-7 signal transduction pathway that represses Bax, the mechanism of Bax translocation, the roles of Bad and Bim, and the trigger for death of adult pro-T cells.

Experimental Procedures

Mice and Cell Lines

Bax-deficient mice, B6.129X1-Bax^{tm1Sjk}, were backcrossed eight generations onto C57BL/6 and are statistically 99.6% C57BL/6-like at all loci not linked to the Bax gene. Bax^{+/-} mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the Animal Production Area, NCI-FCRDC (Frederick, MD). IL-7R-deficient mice, homozygous for the IL7^{tm1lmx} target mutation, B6.129S7-IL7^{tm1lmx}, were purchased from The Jackson Laboratory, backcrossed six times onto C57BL/6, and maintained by homozygous breeding at the Animal Production Area, NCI-FCRDC. IL-7R^{-/-}/Bax^{-/-} mice were bred by crossing mice heterozygous for the Bax^{tm1Sjk} mutation with homozygotes for the IL7^{tm1lmx} mutation to produce offspring heterozygous at both genes that were then mated to generate the double-deficient mice.

The mice, C57BL/6-TgN(Bcl2)36Wehi (Strasser et al., 1990, 1991), were transgenic for expression of Bcl-2 in both T and B lymphocytes. These mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the National Institutes of Health.

The IL-7^{-/-}/Rag^{-/-} mice were provided by R. Murray (EOS Biotechnology, San Francisco, CA) and a colony maintained through homozygous breeding at the Animal Production Area, NCI-FCRDC (Frederick, MD). Mice homozygous for the Rag1^{tm1Mom} mutation, B6.129S7-Rag1^{tm1Mom}, were purchased from The Jackson Laboratory (Bar Harbor, ME) and a colony established through homozygous breeding at the Animal Production Area, NCI-FCRDC (Frederick, MD), as were the C57BL/6 and B6 SCID/SCID mice. To produce timed pregnancy, mice were mated overnight and plugs checked the following day, which was designated as day 1 of gestation. At day 14 of gestation, mothers were killed by CO₂ asphyxiation and embryos by chilling on ice. Fetal thymus lobes were removed under a dissecting microscope.

All mice were housed under pathogen-free conditions. Animal care was provided in accordance with the procedures outlined in the “Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 86-23, 1985). Genotyping of the mice was performed using two sets of polymerase chain reactions (PCR) for identifying wild-type (wt) and knockout alleles of Bax and IL-7R, following protocols available from The Jackson Laboratory (Bar Harbor, ME). DNA was extracted from tissue derived from tail clips using a commercially available extraction kit (Nucleospin, Clontech).

The two cytokine cell lines employed, the IL-7-dependent thymic cell line, D1, and the IL-3-dependent pro-B cell line, FL5.12A, have been previously described (Khaled et al., 2001a, 2001b).

Histology and Immunohistochemistry

Thymic lobes were dissected and fixed in 10% neutral buffered formalin. For immunohistochemistry, paraffin blocks were sectioned, slides deparaffinized, and prepared by a 15 min methanol block and microwave pretreatment. After blocking with 5% normal serum for 20 min, slide sections were incubated with primary antibody and anti-CD3 antibody (clone A0452, Dako) and washed in PBS, and biotinylated rabbit secondary antibody (Vector) was added. Following PBS washing, slides were treated with ABC reagent (biotin/avidin complex) (Vector) and rinsed, and DAB substrate (1 DAB tablet (Sigma) in 15 ml PBS and 12 μ l 30% H₂O₂) was added. Slides were rinsed and counterstained in Gill's hematoxylin and then stained blue with lithium carbonate.

Isolation and Treatment of Thymocytes and Pro-T Cells

Thymocytes were isolated from thymic lobes by gentle disruption with a micropipette after treatment with 0.2% collagenase (Sigma) (Kim et al., 1998). To isolate triple-negative (TN) pro-T cells by negative separation, total thymocytes were first resuspended in complete medium, RPMI + 10% FBS, supplemented with 50 ng/ml IL-7 (Pe-

protech), at 10^7 cells/ml. To each tube, 5 μ g of rat anti-mouse antibodies, anti-CD3 (clone 17A2), anti-CD4 (clone GK1.5), and anti-CD8 (clone 53-6.7) (Pharmingen) was added. After incubation, cells were washed and resuspended with goat anti-rat IgG antibody coated magnetic beads (BioSource International), following manufacturer's protocol. Cells were magnetically separated to remove the CD3⁺, CD4⁺, or CD8⁺-positive cells. This was repeated twice. The recovered supernatant contained the TN pro-T cells that represented on the average 2%–3% of the starting thymocyte population. To confirm purity of negatively selected pro-T cells, staining with antibodies for CD3, CD4, and CD8 was performed as described below with an average of 90% TN pro-T cells recovered.

To analyze IL-7 withdrawal-induced death, pro-T cells were placed in 96-well U-bottom culture plates (Costar) at a concentration of 5×10^5 cells in 100 μ l of medium with or without 50 ng/ml IL-7. After time points specified in figure legends, aliquots of cells were removed, total cells microscopically counted, and viabilities determined by trypan blue exclusion (MediaTech). For analysis of pro-T cell subset distribution, flow cytometry was performed as described below.

Flow Cytometry

For analysis of thymic development, 10^5 cells were collected into PBS with 5% FBS and stained with saturating amounts of anti-CD3-FITC (clone 145-2C11), anti-CD4-PE (clone RM4-5), and anti-CD8-cyChrome (clone 53-6.7) (Pharmingen). For analysis of pro-T cell subsets, 10^5 cells pro-T cells were collected and stained with anti-CD25-FITC (clone 7D4) and anti-CD44-PE (clone IM7) (Pharmingen). After incubation, cells were washed and fixed in 2% paraformaldehyde solution. List mode data were acquired on a FACScan flow cytometer (Becton Dickinson, Palo Alto, CA) using PC lysis software. Dead cells were excluded by forward and side scatter gating.

For analysis of TN thymocytes, 10^5 thymocytes were stained with anti-CD3-FITC (clone 145-2C11), anti-CD4-FITC (clone RM4-5), and anti-CD8-FITC (clone 53-6.7) (Pharmingen), followed by staining with anti-CD44-PE (clone IM7) and anti-CD90.2-biotin (Thy 1.2) (clone 53-2.1), followed by streptavidin-cyChrome (Pharmingen). All the FITC-positive cells were excluded by gating, and the FITC-negative cells were analyzed for CD44 and Thy-1 expression.

Detection of $\gamma\delta$ T Cells and Rearrangement of the TCR γ Locus

For detection of $\gamma\delta$ T cells, splenic cells were isolated from freshly harvested spleens by manual teasing. Red blood cells (RBCs) were lysed in 0.84% ammonium chloride. Approximately 10^5 splenocytes were stained with saturating amounts of anti-TCR β -FITC (clone H57-597) and anti-TCR $\gamma\delta$ (clone GL3) (Pharmingen) and analyzed for expression by flow cytometry as previously described.

Detection of TCR γ locus rearrangement was performed by PCR amplification, using methods previously published (Candeias et al., 1997b; Durum et al., 1998). In brief, DNA was extracted from spleen cells and approximately 0.25 μ g of DNA was used for each PCR reaction. Thirty cycles of amplifications were performed, each cycle consisting of 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 1 min of elongation at 72°C. Primers used were V γ 2: 5'-CATGGGAAGTTGGAGCAACCTGAAATATC-3' and J γ 1: 5'-GCTTC GTCTTCTTCTCCAAGGATA-3'. Separation of PCR products was previously described (Candeias et al., 1997; Durum et al., 1998).

Thymic Reconstitution Experiments

Bone marrow (BM) donor cells (recovered by flushing cells from femurs) or fetal liver (FL) cells were isolated from Bax^{-/-} mice. Donor BM or FL cells were resuspended at 5×10^7 cells/ml in complete medium. Recipient mice, Rag^{-/-}, or IL-7^{-/-}/Rag^{-/-} double-deficient mice, were irradiated at 400 rads. Recipient mice were also pretreated with Asial-GM-1. Approximately $1-2 \times 10^6$ cells were injected into the tail vein of each recipient mouse. Mice were placed on acid water and antibiotics, then sacrificed for evaluation of thymic content 4–5 weeks later.

Fetal thymic organ cultures were performed as previously described (Lee et al., 2001). In brief, BM cells from an IL-7R^{-/-}/Bax^{-/-} double-deficient mouse, as well as from wt, IL-7R^{-/-}, and Bax^{-/-} deficient mice (harvested as described above), were used to reconstitute irradiated (30 Gy) fetal thymuses, from day 14 embryos, in a

hanging drop culture—10,000 BM cells used per thymus. After several days to allow thymic reconstitution, five to eight fetal thymus lobes were placed on a nylon screen (100 mesh) that was supported by a plastic cylinder in a 24-well tissue culture plate containing complete medium. The cultures were incubated at 37°C in 5% CO₂ and fed with complete medium every 2 to 3 days. After 1 week, thymuses were harvested, and single-cell suspensions were made and analyzed.

Western Blots for Proapoptotic Proteins

Whole-cell lysates were made from thymocytes, mouse splenocytes, D1, or FL5.12A pro-B cells in a modified RIPA buffer containing phosphatase inhibitors and protease inhibitors as previously described (Khaled et al., 2001a). Cell lysates were spun at 20,000 g at 4°C, and the recovered supernatant was used for protein analysis. For detection of Bax, Bad, and Bak protein by Western blot, cell equivalent samples (20 μ l containing approximately 40 μ g protein) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Tris-glycine gels (Invitrogen) and transferred to 0.2 μ M polyvinylidene difluoride (PVDF) membranes (Invitrogen). Blots were probed with a rabbit polyclonal antisera specific for the amino terminal of Bax (N-20, Santa Cruz), a mouse monoclonal antibody to detect Bad phosphorylated at serine 112 (7E11, Cell Signaling), a mouse monoclonal antibody for Bim (Ab-1, Oncogene), or a mouse monoclonal antibody for Bak (Ab-2, Oncogene) followed by the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (Santa Cruz) and then visualized by ECL (Pierce) following the manufacturer's protocol.

Acknowledgments

We wish to acknowledge J. Ashwell, M.D. for his insights regarding the effects of steroids in adult mice. We are also grateful to J. Oppenheim, M.D. and W. Leonard, M.D. for their comments on the manuscript. This publication has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under contract No. NO1-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

Received: April 18, 2002

Revised: August 22, 2002

References

- Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R., and Weissman, I.L. (1997). Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell* 89, 1033–1041.
- Bouillet, P., Purton, J.F., Godfrey, D.I., Zhang, L.C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J.M., and Strasser, A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415, 922–926.
- Brugnera, E., Bhandoora, A., Cibotti, R., Yu, Q., Guinter, T.I., Yamashita, Y., Sharrow, S.O., and Singer, A. (2000). Coreceptor reversal in the thymus: signaled CD4⁺ thymocytes initially terminate CD8 transcription even when differentiating into CD8⁺ T cells. *Immunity* 13, 59–71.
- Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96, 857–868.
- Candeias, S., Muegge, K., and Durum, S.K. (1997a). IL-7 receptor and VDJ recombination: trophic versus mechanistic actions. *Immunity* 6, 501–508.
- Candeias, S., Peschon, J.J., Muegge, K., and Durum, S.K. (1997b). Defective T-cell receptor gamma gene rearrangement in interleukin-7 receptor knockout mice. *Immunol. Lett.* 57, 9–14.
- Cao, X., Shores, E.W., Hu-Li, J., Anver, M.R., Kelsall, B.L., Russell, S.M., Drago, J., Noguchi, M., Grinberg, A., and Bloom, E.T. (1995).

- Defective lymphoid development in mice lacking expression of the common cytokine receptor chain. *Immunity* 2, 223–238.
- Datta, S.R., Katsov, A., Hu, L., Petros, A., Fesik, S.W., Yaffe, M.B., and Greenberg, M.E. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol. Cell* 6, 41–51.
- Deckwerth, T.L., Elliott, J.L., Knudson, C.M., Johnson, E.M.J., Snider, W.D., and Korsmeyer, S.J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* 17, 401–411.
- del-Peso, L., Gonzalez, G.M., Page, C., Herrera, R., and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278, 687–689.
- Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L., and Coffey, P.J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr. Biol.* 10, 1201–1204.
- Dijkers, P.F., Birkenkamp, K.U., Lam, E.W., Thomas, N.S., Lammers, J.W., Koenderman, L., and Coffey, P.J. (2002). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J. Cell Biol.* 156, 531–542.
- Downward, J. (1999). How BAD phosphorylation is good for survival. *Nat. Cell Biol.* 1, E33–E35.
- Durum, S.K., Candeias, S., Nakajima, H., Leonard, W.J., Baird, A.M., Berg, L.J., and Muegge, K. (1998). Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. *J. Exp. Med.* 188, 2233–2241.
- Griffiths, G.J., Corfe, B.M., Savory, P., Leech, S., Esposti, M.D., Hickman, J.A., and Dive, C. (2001). Cellular damage signals promote sequential changes at the N-terminus and BH-1 domain of the pro-apoptotic protein Bak. *Oncogene* 20, 7668–7676.
- Hofmeister, R., Khaled, A.R., Benbernou, N., Rajnavolgyi, E., Muegge, K., and Durum, S.K. (1999). Interleukin-7: physiological roles and mechanisms of action. *Cytokine Growth Factor Rev.* 10, 41–60.
- Hsu, S.Y., Kaipia, A., McGee, E., Lomeli, M., and Hsueh, A.J. (1997). Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc. Natl. Acad. Sci. USA* 94, 12401–12406.
- Khaled, A.R., Kim, K., Hofmeister, R., Muegge, K., and Durum, S.K. (1999). Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proc. Natl. Acad. Sci. USA* 96, 14476–14481.
- Khaled, A.R., Moor, A.N., Li, A., Kim, K., Ferris, D.K., Muegge, K., Fisher, R.J., Fliegel, L., and Durum, S.K. (2001a). Trophic factor withdrawal: p38 mitogen-activated protein kinase activates NHE1, which induces intracellular alkalization. *Mol. Cell Biol.* 21, 7545–7557.
- Khaled, A.R., Reynolds, D.A., Young, H.A., Thompson, C.B., Muegge, K., and Durum, S.K. (2001b). Interleukin-3 withdrawal induces an early increase in mitochondrial membrane potential unrelated to the Bcl-2 family. Roles of intracellular pH, ADP transport, and F(0)F(1)-ATPase. *J. Biol. Chem.* 276, 6453–6462.
- Kim, K., Lee, C.K., Sayers, T.J., Muegge, K., and Durum, S.K. (1998). The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. *J. Immunol.* 160, 5735–5741.
- Knudson, C.M., and Korsmeyer, S.J. (1997). Bcl-2 and Bax function independently to regulate cell death. *Nat. Genet.* 16, 358–363.
- Knudson, C.M., Tung, K.S., Tourtellotte, W.G., Brown, G.A., and Korsmeyer, S.J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* 270, 96–99.
- Kondo, M., Akashi, K., Domen, J., Sugamura, K., and Weissman, I.L. (1997). Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common γ chain-deficient mice. *Immunity* 7, 155–162.
- Lee, C.K., Kim, K., Welniak, L.A., Murphy, W.J., Muegge, K., and Durum, S.K. (2001). Thymic emigrants isolated by a new method possess unique phenotypic and functional properties. *Blood* 97, 1360–1369.
- Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., et al. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol. Cell* 6, 1389–1399.
- Maki, K., Sunaga, S., Komagata, Y., Kodaira, Y., Mabuchi, A., Karsuyama, H., Yokomuro, K., Miyazaki, J.I., and Ikuta, K. (1996). Interleukin 7 receptor-deficient mice lack $\gamma\delta$ T cells. *Proc. Natl. Acad. Sci. USA* 93, 7172–7177.
- Maraskovsky, E., O'Reilly, L.A., Teepe, M., Corcoran, L.M., Peschon, J.J., and Strasser, A. (1997). Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1^{−/−} mice. *Cell* 89, 1011–1019.
- Maraskovsky, E., Peschon, J.J., McKenna, H., Teepe, M., and Strasser, A. (1998). Overexpression of Bcl-2 does not rescue impaired B lymphopoiesis in IL-7 receptor-deficient mice but can enhance survival of mature B cells. *Int. Immunol.* 10, 1367–1375.
- Matsuzaki, Y., Nakayama, K., Tomita, T., Isoda, M., Loh, D.Y., and Nakachi, H. (1997). Role of bcl-2 in the development of lymphoid cells from the hematopoietic stem cell. *Blood* 89, 853–862.
- Mok, C.L., Gil-Gomez, G., Williams, O., Coles, M., Taga, S., Tolaini, M., Norton, T., Kioussis, D., and Brady, H.J. (1999). Bad can act as a key regulator of T cell apoptosis and T cell development. *J. Exp. Med.* 189, 575–586.
- Nakajima, H., and Leonard, W.J. (1999). Role of Bcl-2 in alpha beta T cell development in mice deficient in the common cytokine receptor gamma-chain: the requirement for Bcl-2 differs depending on the TCR/MHC affinity. *J. Immunol.* 162, 782–790.
- Namen, A.E., Schmierer, A.E., March, C.J., Overell, R.W., Park, L.S., Urdal, D.L., and Mochizuki, D.Y. (1988). B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *J. Exp. Med.* 167, 988–1002.
- Oltvai, Z.N., Millman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74, 609–619.
- Patel, T.D., Jackman, A., Rice, F.L., Kucera, J., and Snider, W.D. (2000). Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. *Neuron* 25, 345–357.
- Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., Ware, C.B., et al. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180, 1955–1960.
- Sasaki, T., Irie-Sasaki, J., Jones, R.G., Oliveira-dos-Santos, A., Stanford, W.L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., et al. (2000). Function of PI3K γ in thymocyte development, T cell activation, and neutrophil migration. *Science* 287, 1040–1046.
- Strasser, A., Harris, A.W., Vaux, D.L., Webb, E., Bath, M.L., Adams, J.M., and Cory, S. (1990). Abnormalities of the immune system induced by dysregulated bcl-2 expression in transgenic mice. *Curr. Top. Microbiol. Immunol.* 166, 175–181.
- Strasser, A., Harris, A.W., and Cory, S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 67, 889–899.
- Vacchio, M.S., and Ashwell, J.D. (2000). Glucocorticoids and thymocyte development. *Semin. Immunol.* 12, 475–485.
- Vander, H.M., Plas, D.R., Rathmell, J.C., Fox, C.J., Harris, M.H., and Thompson, C.B. (2001). Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol. Cell Biol.* 21, 5899–5912.
- Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 75, 229–240.
- Venkitaraman, A.R., and Cowling, R.J. (1994). Interleukin-7 induces

the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. *Eur. J. Immunol.* 24, 2168–2174.

von-Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E., and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J. Exp. Med.* 181, 1519–1526.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292, 727–730.

Wen, R., Wang, D., McKay, C., Bunting, K.D., Marine, J.C., Vanin, E.F., Zambetti, G.P., Korsmeyer, S.J., Ihle, J.N., and Cleveland, J.L. (2001). Jak3 selectively regulates Bax and Bcl-2 expression to promote T-cell development. *Mol. Cell. Biol.* 21, 678–689.

Yin, X.M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K.A., and Korsmeyer, S.J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 400, 886–891.

Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R., and Thompson, C.B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev.* 15, 1481–1486.